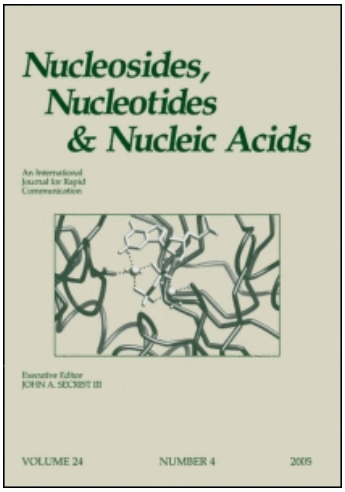

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Vern L. Schramm[a]
[a] Albert Einstein College of Medicine, Yeshiva University, Bronx, New York, USA

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Immucillins as Antibiotics for T-Cell Proliferation and Malaria

Vern L. Schramm*

Albert Einstein College of Medicine, Yeshiva University, Bronx, New York, USA

ABSTRACT

The genetic deficiency of human PNP causes a specific immunodeficiency by inducing apoptosis in dividing T-cells. Powerful inhibitors of PNP have been designed from the experimental determination of the transition state structure of PNPs. The Immucillins are transition state analogue inhibitors with K_d values as low as 7 pM. In the presence of deoxyguanosine the Immucillins kill activated human T-cells but not other cell types. The Immucillins are orally available and of low toxicity to mice. Immucillins also inhibit PNP from *Plasmodium falciparum*. Parasites cultured in human erythrocytes are killed by purine starvation in the presence of Immucillins and can be rescued by hypoxanthine.

Key Words: Purine nucleoside phosphorylase; Immucillin-H; DADMe-Immucillin-H; T-cell proliferation; Malaria; Purine salvage Transition state; Transition state analogues.

INTRODUCTION: DISCOVERY AND METABOLIC ROLE OF PNP

The first report of a human patient genetically deficient in purine nucleoside phosphorylase (PNP) was made by Dr. Eloise Giblett in 1975.[1] The patient had

*Correspondence: Vern L. Schramm, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Ave., Bronx, NY 10461, USA.

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the unusual phenotype of being normal at birth, but gradually lost T-cell function over a period of one to two years, while being normal in other respects, including B-cell function. The T-cell immunodeficiency was distinct from the previous severe combined immunodeficiency (SCID) attributed to adenosine deaminase deficiency, discovered earlier by Dr. Giblett.[2] The concentration of dGTP is elevated in cells of PNP deficient patients, closely related to the observation of elevated dATP in cells of SCID children.[3] Phosphorylation of deoxyguanosine by the deoxycytidine kinase (dCK) of activated T-cells and accumulation of dGTP is now known to induce apoptotic cell-death.[4] Under normal conditions, there is no detectable deoxyguanosine in human plasma or urine because of the highly active PNP in many human tissues, including micromolar concentrations in erythrocytes. In children with PNP deficiency, deoxyguanosine levels increase to 10 μM in plasma and 2 to 4 mmol/g creatinine in urine.[4] Profound deficiency of PNP activity is required to induce T-cell immunodeficiency, since even a few percent of normal catalytic activity permits T-cell function.

A single-enzyme genetic deficiency that regulates T-cell division attracted the interest of the pharmaceutical industry.[5] Human diseases that might be ameliorated by controlling T-cell function include T-cell leukemia and lymphoma, tissue transplant rejection and the T-cell autoimmunities including psoriasis, inflammatory bowel disease, rheumatoid arthritis and insulin-dependent juvenile diabetes. By 1998, over thirty patents had been issued for PNP inhibitors to control T-cell activity.[5] Several inhibitors entered clinical trials but none were able to recreate the human phenotype of PNP deficiency.

TRANSITION STATE STRUCTURE OF PNPs

Transition state theory indicates that perfect mimics of the transition state will bind tighter than substrate by the factor of enzymatic rate enhancement, typically 10^{10} to 10^{15}. PNP catalyzes the reactions: 6-oxypurine-α-D-(deoxy)riboside + $PO_4 \leftrightarrow$ 6-oxypurine + α-D-(deoxy)ribose-1-PO_4, where deoxyguanosine is the important substrate for the human genetic disorder of PNP. Enzymatic transition states can be investigated by kinetic isotope effects and studies with bovine PNP indicated a transition state structure with a N9–C1′ bond length of 1.77 Å but only van der Waals contacts at 3.0 Å to the attacking anion (Fig. 1).[6] In transition state studies arsenate was used as the nucleophile, since phosphate prevented measurement of intrinsic kinetic isotope effects.[6] Two features that distinguish the transition state from the substrate molecule are the partial positive charge on the ribosyl group and the elevated pK_a at N7. Transition state analogues must be chemically stable mimics of the transition state, and these features could be reproduced in a stable molecule with the structure of Immucillin-H (Fig. 1).

DESIGN AND SYNTHESIS OF IMMUCILLIN-H

Based on the transition structure of bovine PNP, Immucillin-H was synthesized starting with D-gulonolactone to introduce the four achiral centers, and this synthetic approach has been refined to optimize separate syntheses of protected iminoribitol and

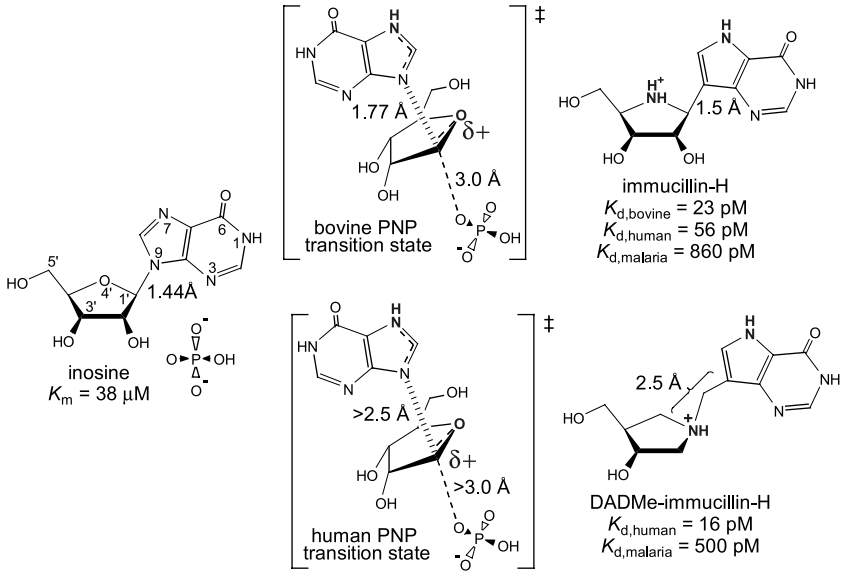

Figure 1. The substrate, transition states and Immucillin transition state analogues for bovine and human PNPs. Inosine is shown with its atomic numbering and the K_m value for the cloned and overexpressed human enzyme. This numbering system is also used to discuss the Immucillins in the text, although it is not in conformity with IUPAC nomenclature for these compounds. Kinetic isotope effects were used to establish the transition state dimensions for both enzymes. The exact structure for human PNP is still in progress. (From Ref. [14].) The K_d values shown for Immucillin-H and DADMe-Immucillin-H (4′-*D*eaza-1′-*A*za-2′-*D*eoxy-9,1′-*M*ethylene Immucillin-H) are dissociation constants following the completion of slow-onset, tight-binding inhibition. Interatomic distances are shown both for the transition states and the transition state analogues.

deazapurine, followed by condensation of the two halves.[7] Immucillin-H was found to be a 23 pM transition state analogue inhibitor of bovine PNP.[8] Transition state analogues are powerful inhibitors of their cognate enzymes, and usually bind in a two-step process. Immucillin-H demonstrates slow-onset, tight-binding inhibition where the initial complex is bound 2000 fold weaker than the complex formed after an enzymatic conformational change. Bovine PNP is a homotrimer, and an unusual feature of its inhibition is that the enzyme is completely inhibited by binding of one molecule of Immucillin-H per trimer, suggesting sequential catalysis.[8]

ENZYMATIC CONTACTS TO BOUND IMMUCILLIN-H

In the presence of Immucillin-H and PO_4, bovine PNP crystallized to give a 1.5 Å resolution X-ray structure of the complex.[9] Comparing complexes with substrates and products reveals that at least six new H-bonds are formed with Immucillin-H and PO_4 at the catalytic site, readily explaining the 10^6-fold ratio of K_m/K_d for Immucillin-H. A family of Immucillin analogues has been synthesized to explore the energetics of Immucillin interactions. The results confirmed that Immucillin-H was the optimum

structure for binding and demonstrated that the essential features to capture transition state binding forces are the oxacarbenium ion mimic and the H-bonds to N7 of the purine ring.[10]

T-CELL SPECIFICITY FOR IMMUCILLIN-H

Immucillin-H was tested in cultured cells and found to generate a PNP-deficiency state similar to that found in human genetic PNP deficiency. Human T-cell cancer CEM and MOLT-4 cell lines were sensitive to suppression by Immucillin-H only in the presence of deoxyguanosine. Other human cell lines, including colon (Geo), B-cell (BL-2) and 17 other mammalian non T-cell lines were insensitive to these treatments, even at 10^{-5} M Immucillin-H while the IC_{50} values for sensitive cells were 10^{-9} to 10^{-8} M.[11] Resting human T-cells are insensitive to Immucillin-H and deoxyguanosine, but when normal T-cells are stimulated to divide with IL-2 and autologous antigens, they become as sensitive as T-cell cancer lines. Treatment of rapidly dividing T-cells with Immucillin-H and deoxyguanosine causes the T-cells to undergo apoptosis.

BIOAVAILABILITY, IMMUNOSUPPRESSION AND TOXICITY

Recent studies have revealed that Immucillin-H[a] is an effective PNP inhibitor in mice and primates by either oral or intravenous administration.[12,13] In a mouse model of xenogeneic graft vs. host disease, Immucillin-H was as effective as cyclosporin in extending mouse survival, and the combination of cyclosporin and Immucillin-H was more effective than treatment with either agent alone.[12] Studies in primates indicate that toxicity is low and phase I/II clinical trials have been initiated in patients with T-cell leukemia.[13]

DADMe-IMMUCILLIN-H AS A HUMAN PNP INHIBITOR

Immucillin-H was designed to match the transition state of bovine PNP and it binds less tightly to human and malarial PNPs, with K_d values of 56 and 860 pM, respectively.[14] This result indicates that the transition states of the PNP isozymes differ, since transition state analogues capture binding energy on the basis of transition state recognition. Kinetic isotope effect measurements of human PNP indicate a more dissociated transition state, with the bond to the leaving group > 2.5 Å and to the nucleophile > 3.0 Å[14] (Fig. 1). On the basis of this result, our chemistry group synthesized DADMe-Immucillin-H and its 9-deazaguanine analogue DADMe-Immucillin-G.[15] These analogues have the nitrogen of the ribooxacarbenium ion mimic moved to the 1′-position to more closely mimic the cationic charge location of the more dissociated transition state and a methylene bridge between the ribosyl analogue

[a]Immucillin-H is being developed under the trade name BCX-1777, see Refs. [4,12,13], and is now called Fovodosine (see Ref. [20]).

and the 9-deazapurine to more closely mimic the geometry of the human PNP transition state. DADMe-Immucillin-H and DADMe-Immucillin-G are 16 and 7 pM inhibitors of human PNP, respectively. With the K_m value of 38 μM, the K_m/K_d ratio for DADMe-Immucillin-G with human PNP is 5,400,000.

BIOLOGICAL EFFICIENCY OF IMMUCILLIN-H AND DADMe-IMMUCILLIN-H IN MOUSE MODELS

Feeding a single oral dose of Immucillins to mice, followed by sampling of PNP in the blood provides a convenient assay for oral availability and the biological half-life for inhibition of PNP. Erythrocytes in mice and humans contain micromolar concentrations of PNP and this activity must be inhibited more than 99% to permit deoxyguanosine accumulation and T-cell inhibition. A single oral dose of 0.1 μmol (27 μg, 0.8 mg/kg) Immucillin-H causes a loss of blood PNP activity with a $t_{1/2}$ of 14 min and regain of 50% of normal activity after 100 hr (4.2 days). The same dose of DADMe-ImmH results in loss of PNP activity with a $t_{1/2}$ of 10 min and regain of 50% of normal activity after 275 hr (11.5 days).[16] The lifetime of mouse erythrocytes is reported to be 25 days, therefore 50% of the erythrocyte pool is regenerated after 12.5 days. The biological effectiveness of DADMe-Immucillin-H is sufficient in that a single oral dose causes near-complete inhibition of the target enzyme for the lifetime of circulating erythrocytes. This is the ultimate goal of inhibitor design, to permit complete inhibition of the target enzyme by single oral dose agents.

IMMUCILLIN-H AND PURINE PATHWAYS IN MALARIA

Plasmodium falciparum is a purine auxotroph and it derives purines for growth and division from the purine pool of the erythrocyte during its life-cycle in humans. It is known that hypoxanthine is a major source of these purines, through the proposed pathway: adenosine → inosine → hypoxanthine → IMP → → AMP + GMP, using the sequential actions of adenosine deaminase, PNP, HGXPRTase and the purine synthetic pathway for AMP and GMP production. We tested the hypothesis that blocking PNP would interrupt this pathway to prevent purine salvage and prevent *P. falciparum* growth. Exposure of *P. falciparum* cultures in human erythrocytes to Immucillin-H causes parasite cell death within 24 hr if there is no addition of exogenous purines to the culture medium.[17] The sensitivity of *P. falciparum* to Immucillin-H is in the same concentration range (10^{-9} to 10^{-8} M) that induces apoptosis in rapidly-dividing T-cells. Addition of hypoxanthine (but not inosine or adenosine) prevents the action of Immucillin-H, demonstrating the site of action to be at PNP. The efficiency of Immucillin-H in targeting the purine salvage pathway is surprising since alternative salvage pathways have been proposed.[18] The recently released genome sequence of *P. falciparum* provides insights into this pathway, and indicates that there are no enzymes of purine salvage to by-pass the salvage sequence shown above.[19] This potential application of Immucillin-H to malaria is highly pertinent since Immucillin-H is currently in clinical trials.

CONCLUSION AND SUMMARY

Transition state theory has led to the production of the Immucillins, the most powerful inhibitors yet designed for PNP. These inhibitors have potential for treatment of T-cell cancers, autoimmune disorders, tissue transplant rejection and malaria. We predict that other applications of transition state theory will provide new and powerful transition state analogues for a variety of biomedical applications. In the DADMe-Immucillins, the ultimate goal of inhibitor design has been accomplished for the mouse model of PNP inhibition by providing near-complete inhibition of the target enzyme for the lifetime of the target cell with a single oral dose.

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